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SEROLOGICAL GROUPING OF HEMOLYTIC
STREPTOCOCCI BY COUNTER-
IMMUNOELECTROPHORESIS

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Serological Grouping of Hemolytic Streptococci by Counter-Immunoelectrophoresis

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The counter-immunoelectrophoresis (CIE) method of grouping streptococci was more sensitive than the capillary precipitin method. The precipitate was easier to read, and the test was simple to perform and required fewer reagents. An autoclave-Pronase-B extraction procedure is described and was found superior to either acid, Pronase-B, or the autoclave extraction procedures for recovering groupable polysaccharide from different strains of streptococcal groups A, B, C, D, F, and G. Data obtained from over 400 strains indicate that the sensitivity of the CIE and a more efficient extraction of groupable polysaccharide provides a simple method for rapid diagnosis of streptococcal infections.

A rapid method for differentiating group A streptococci from other beta-hemolytic streptococci and/or other agents causing upper respiratory infections has been the goal of microbiologists since the relationship between group A streptococci and rheumatic fever was established. A presumptive test using a bacitracin-saturated filter paper disk has been described (11, 12) and is commonly employed in most diagnostic laboratories. Although this method has simplified the approach for rapid differentiation of group A beta-hemolytic streptococci from other streptococci, technical problems may have contributed to improper interpretations resulting in inadequate or needless treatment of patients (1, 8). From the standpoint of providing maximal patient care, as far as the consequences of streptococcal infections are concerned, specific streptococcal grouping of the beta-hemolytic streptococci is the most accurate. Such specific grouping of beta-hemolytic streptococci requires the extraction of group-specific carbohydrates and subsequent precipitin testing (10) or the use of fluorescent antibody techniques with group-specific labeled antiserum (13). The latter test requires expensive equipment and well-trained technicians. In small laboratories, where isolation and grouping of streptococci may occur infrequently, the use of the Lancefield acid extraction method may be too complicated a procedure to be reliable. In addition, the precipitate in the capillary tube may be hard to interpret because of either a dirty capillary tube or a slightly cloudy antiserum. Consequently, errors in grouping strep-

tococci in laboratories with limited capabilities may occur with unacceptable frequency.

The counter-immunoelectrophoresis technique (CIE) has been shown to provide a very sensitive and easily interpreted procedure for detecting *Australia* (9), meningococcal (6), pneumococcal (4), and *Haemophilus influenzae* type B antigens (2). It has also been used to group or type meningococci and pneumococci (6, 7). Recent studies indicate that the method may also be useful in grouping streptococci (E. A. Edwards and G. L. Larson, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 76, 1973; reference 3).

The present study was undertaken to determine the sensitivity, specificity, and reliability of CIE compared with the capillary tube precipitin test in differentiating and grouping beta-hemolytic streptococci. In addition, an improved method of extracting groupable polysaccharide from various strains of streptococci is described which was from 4 to 32 times more efficient than the conventional acid extraction procedure.

MATERIALS AND METHODS

Counter-immunoelectrophoresis. The CIE method has been previously described (6), except 0.05 M barbital buffer (pH 8.6) was used in both the agarose and electrode vessels. Glass slides were coated with 1% agarose in barbital buffer (0.05 M, pH 8.6; Kallestad Laboratories, Minneapolis, Minn.). Wells (3-mm diameter) were cut in parallel rows 1.5 mm apart. Streptococcal extract (10 µliters) was placed in the wells on the cathode (negative) side of the plate, and specific grouping serum (10 µliters) was added to

the opposite wells. Electrophoresis was carried out for 1 h by using 12 mA per glass slide (3.25 by 4 inches [approximately 8.13 by 10 cm]; Kodak slide) and 3 mA per microscope slide. Visualization of the precipitin bands was made by using a viewer (Kallestad Laboratories) or a 7× hand lens.

Laboratory strains of beta-hemolytic streptococci were used in the preliminary evaluation of the CIE method to detect group-specific streptococcal antigen compared with the capillary precipitin tube test as the accepted standard of reference. The number of strains and their groups were 15 A, 8 B, 4 C, 3 D, 3 F, and 4 G. Each strain was streaked onto a blood agar plate, and the plates were incubated for 18 to 24 h at 37°C under 10% CO₂. A single colony was transferred into 30 ml of Todd-Hewitt (T-H) broth (Difco Laboratories, Detroit, Mich.). The broth culture was incubated from 18 to 24 h at 37°C without CO₂. The growth was sedimented by centrifugation and extracted by the Lancefield method (10).

Comparison of the sensitivity of CIE with the capillary precipitin test. To determine whether the CIE test was more sensitive in detecting group-specific polysaccharide than was the capillary precipitin tube test, the following tests were made. A 5-ml sample of an 18- to 24-h T-H broth culture, each of types 1 and 12, was acid-extracted (10). Twofold serial dilutions of the extracts were made in 0.15 M NaCl (saline). Each dilution of each streptococcal type was grouped by the capillary precipitin tube method and by CIE. The highest dilution of each extract that produced a precipitate was considered the "sensitivity of the test procedure."

Determination of yield of group-specific polysaccharide by various extraction methods. The Lancefield acid (10), autoclave (14), and Pronase-B (5) extraction procedures were followed without modification. Extracts were made from 5-ml samples of an 18- to 24-h T-H broth culture of group A, type 12, streptococci. An autoclave-Pronase-B extraction procedure was performed as follows. The growth from the 5-ml sample was sedimented by centrifugation, and the supernatant was decanted. The sediment was then autoclaved for 15 min at 15 lb of steam pressure. After cooling (about 5 min), 0.5 ml of Pronase-B solution containing 20 mg of Pronase-B per ml (no. 53702 Pronase, B grade, Calbiochem, LaJolla, Calif.), prepared in borate buffer (5), was added and mixed, and the sediment-Pronase mixture was incubated at 37°C for 2 h. The material was centrifuged, and the clear supernatant from each of the four extraction procedures was serially diluted in saline. Each dilution was tested for antigen by both the precipitin tube method (10) and by CIE. In another experiment, an 18- to 24-h T-H broth culture was separated into four sets, each set containing 5, 4, 3, 2, 1, 0.75, 0.6, 0.25, and 0.1 ml, respectively. Each tube of the four series of samples was extracted for group-specific polysaccharide by one of the four methods described above. The extracts were tested for streptococcal group-specific antigen by CIE.

Clinical studies. Throat cultures obtained from 163 patients visiting the outpatient clinic at the Naval Hospital, Great Lakes, were tested for beta-hemolytic

streptococci by using the conventional blood agar streak-plate method and for streptococci of groups A, B, C, F, and G by placing the swab into 5 ml of T-H broth in a glass tube (13 by 100 mm; see flow diagram, Fig. 1). The blood agar plate was incubated for 18 to 24 h at 37°C in CO₂. A beta-hemolytic colony was used to inoculate 5 ml of T-H broth and was incubated for 18 to 24 h at 37°C, and the sediment was recovered for grouping. The swab used to inoculate the blood agar plate was placed into 5 ml of T-H broth, incubated at 37°C for 1 h, and then mixed with a Vortex mixer for 10 s. The swab was expressed into the medium by "wringing" the swab on the sides of the tube, and the swab was discarded. The culture was reincubated for 3 h, and the contents were sedimented by centrifugation. The sediments of both the streak-plate isolate and the broth-grown cultures were extracted by the autoclave-Pronase-B method described above, and grouping of the extracts was done by CIE.

Grouping sera for all tests were commercially prepared (CDC and Difco). Antisera used in these experiments from CDC were group A (lot no. 23, 26 February 1968), group B (lot no. 14, 4 November 1968), group C (lot no. 10, 16 February 1970), group D (lot no. 5, 2 November 1966), group E (lot no. 4, 28 October 1964), group F (lot no. 12, 1 November 1971), and G (lot no. 13, 21 January 1970). Antisera from Difco were group A (lot no. 550237) and group C (lot no. 550481).

RESULTS

The results in Table 1 show the correlation between grouping streptococci by the conventional capillary precipitin test and the CIE method. In 266 attempts using five different

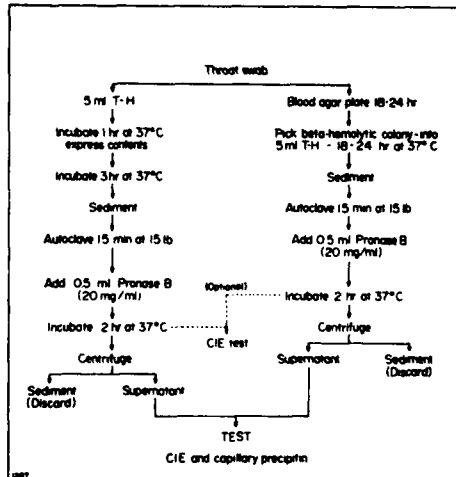


FIG. 1. Flow diagram showing the procedure of grouping streptococci by the conventional method (right) and a 4-h culture method (left).

TABLE 1. Correlation between grouping streptococci by the capillary precipitin tube test and CIE^a

CIE	Precipitin test		
	Positive	Negative	Total
Positive	231	0	231
Negative	0	35	35
Total	231	35	266

^a Group-specific polysaccharide was extracted by the Lancefield acid extraction procedure.

groups of beta-hemolytic streptococci, there was 100% correlation. No effort was made to grade the intensity of either the precipitate in the capillaries or of the band formed by CIE. They were recorded as positive or negative. The data in Fig. 2 demonstrate the specificity of the CIE method of grouping streptococci. In grouping over 357 strains of beta-hemolytic streptococci by this method, there were no cross-reactions.

The data from our experiment to determine whether the CIE method would be more sensitive in detecting streptococcal polysaccharide

than was the capillary procedure are shown in Fig. 3. Increased sensitivity would allow an earlier identification of streptococcal infections. The results indicate that the CIE method is from 4 to 16 times more sensitive in detecting streptococcal polysaccharide than was the capillary method when using the same extracted materials.

The data in Fig. 4 compare the amount of group-specific polysaccharide recovered from a 5-ml sample of an 18- to 24-h broth culture;

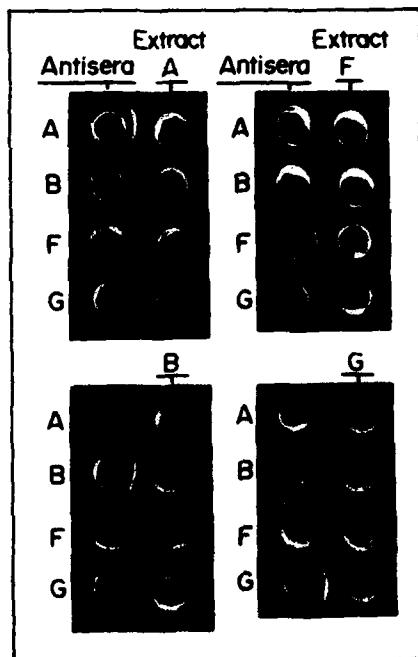


FIG. 2. Demonstration of the specificity of grouping streptococci by the CIE technique.

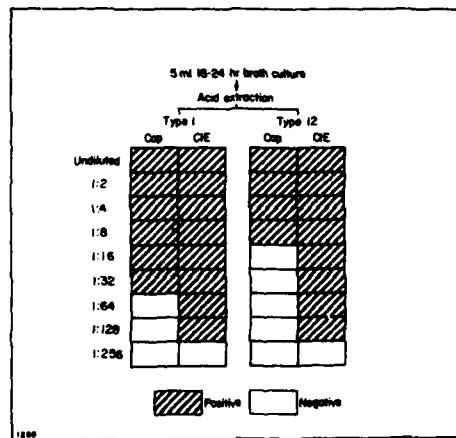


FIG. 3. Comparison of the sensitivity of CIE and capillary precipitin tube test in detecting streptococcal polysaccharide.

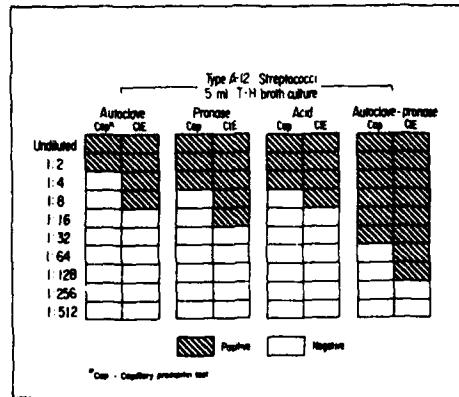


FIG. 4. Comparison of the efficiency of four methods of extracting group-specific polysaccharide. Also demonstrated is the increased sensitivity of the CIE method of grouping streptococci over the capillary precipitin tube test.

each 5 ml was extracted by one of the four procedures described. These data indicate that the autoclave-Pronase-B extraction procedure is from 4 to 16 times more efficient in extracting groupable polysaccharide than are any of the other three procedures.

Figure 5 demonstrates the efficiency of the four extraction procedures in yielding groupable polysaccharide from various amounts of an 18- to 24-h broth culture of five different group A streptococcal strains. It is evident that the autoclave-Pronase-B extraction procedure is consistently more efficient in extracting groupable polysaccharide than are either of the three other methods used.

The data in Table 2 show the results of the clinical studies for identifying streptococci. Of 163 patients studied, 25 were harboring beta-hemolytic streptococci by the conventional

streak-plate method. By transferring a colony to T-H broth and by subsequent extraction for group-specific polysaccharide, the extracts were groupable by either CIE or the capillary precipitin tube test. However, in the 4-h culture method, whereas the CIE detected group-specific polysaccharide in all 25 cases, only 19 out of 25 (76%) gave positive identification with the precipitin tube technique. This difference is significant ($P \approx 0.01$, contingency table, R. Latscha). These results are consistent with our previous experiments in demonstrating that the CIE method is more sensitive in detecting streptococcal polysaccharide than is the capillary precipitin tube procedure. Such sensitivity would allow the laboratory to identify streptococci in approximately 6.5 h if such rapid identifications were necessary.

DISCUSSION

The use of the CIE method of grouping streptococci was demonstrated to be simple, rapid, and reliable. By using the capillary precipitin method as the accepted standard of reference, CIE gave comparable results in all instances and was easier to interpret.

The simple and efficient procedure of extracting groupable polysaccharide, using a combination of autoclave-Pronase-B as detailed above, and the rapid and sensitive method of detecting antigen by the CIE method make this procedure applicable to routine diagnostic services in small, as well as large, laboratories. These procedures eliminate the use of many chemicals, require no pH determinations, and require only small volumes of cultures. The apparatus is currently used routinely in most blood bank laboratories and, therefore, requires no additional expensive equipment. The method is simple, inexpensive (8 cents/extraction), and does not require highly trained technicians. It is conceivable that by using the extraction procedure and CIE to group streptococci, the use of blood agar plates could be eliminated.

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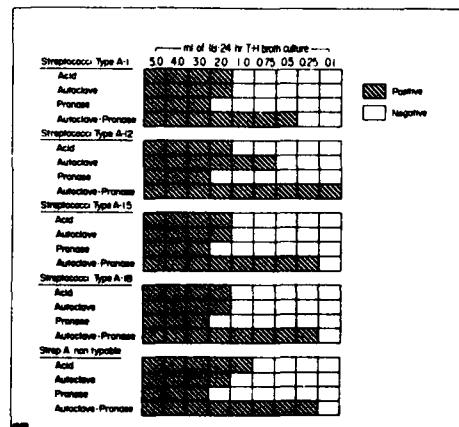


FIG. 5. Comparison of the efficiency of four procedures for extracting group-specific streptococcal polysaccharide from five strains of beta-hemolytic streptococci.

TABLE 2. Sensitivity of CIE compared with the capillary precipitin tube test in 25 positive clinical samples

Method of grouping	Throat swab			
	4-h T-H culture method		18- to 24-h streak-plate method	
	Positive	Negative	Positive	Negative
Precipitin tube test	19	6	25	0
CIE	25	0	25	0
Difference	$P \approx 0.01$			

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